23. Sillén-Tullberg, B. *Evolution* **42,** 293–305 (1988).
24. Fisher, R. A. *The Genetical Theory of Natural Selection* (Clarendon, Oxford, 1930).
25. Müller, F. *Proc. ent. Soc. Lond.* **1879,** 20–29 (1879).
26. Gagliardo, A. & Guilford, T. *Proc. R. Soc. Lond. B* **251,** 69–74 (1993).
27. Sillén-Tullberg, B. *Anim. Behav.* **40,** 856–860 (1990).
28. Stamp, N. E. *Am. Nat.* **115,** 367–380 (1980).

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The PIE-1 protein and germline specification in *C. elegans* embryos

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TOTIPOTENT germline blastomeres in *Caenorhabditis elegans* contain, but do not respond to, factors that promote somatic differentiation in other embryonic cells[1,2]. Mutations in the maternal gene *pie-1* result in the germline blastomeres adopting somatic cell fates[3]. Here we show that *pie-1* encodes a nuclear protein, PIE-1, that is localized to the germline blastomeres throughout early development. During division of each germline blastomere, PIE-1 initially associates with both centrosomes of the mitotic spindle. However, PIE-1 rapidly disappears from the centrosome destined for the somatic daughter, and persists in the centrosome of the daughter that becomes the next germline blastomere. The PIE-1 protein contains potential zinc-finger motifs also found in the mammalian growth-factor response protein TIS-11/NUP475 (refs 4–7). The localization and genetic properties of *pie-1* provide an example of a repressor-based mechanism for preserving pluripotency within a stem cell lineage.

In the development of all animal embryos, certain cells must remain totipotent to form the reproductive cells, or germ cells, for the next generation. The germ cells of the nematode *C. elegans* arise from a sequence of unequal divisions during early embryogenesis[8] (Fig. 1). After each of these divisions, one daughter will produce only somatic cell types, such as muscle cells or neurons; this daughter can be described as a somatic blastomere. The other daughter will produce germ cells in addition to somatic cells, and thus can be described as a germline blastomere.

Studies on cell-fate determination in *C. elegans* embryos have shown that some maternally expressed factors that function in somatic development also are present in the germline blastomeres[1,2]. For example, development of the somatic blastomere in a four-cell embryo, EMS, but not the sister germline blastomere P_2 requires the maternally expressed transcription factors SKN-1 (refs 9, 10) and POP-1 (ref. 2), although these proteins are present in P_2 at the same levels as in EMS[1,2]. P_2 seems to be prevented from responding to these factors by *pie-1*(+) activity[2,3]. The *pie-1* gene is expressed maternally; in *pie-1* mutant embryos, the P_2 blastomere does not produce germ cells, and instead undergoes a pattern of somatic differentiation similar to a wild-type EMS blastomere[3]. In *pie-1; skn-1* double mutants, the germline blastomere in eight-cell stage embryo, P_3, does not produce germ cells and instead undergoes somatic differentiation similar to a wild-type somatic C blastomere[3]. These results suggest that *pie-1*(+) activity in wild-type development prevents P_2 and P_3 from responding to factors that determine the EMS and C fates, respectively.

In initial experiments to clone the *pie-1* gene, we found that the genetic position of *pie-1* coincided with a region of the physical map of the *C. elegans* genome for which there were no available genomic clones or sequences. We therefore screened for rare, spontaneous *pie-1* alleles in a strain with a high frequency of transposon mutagenesis. A single, transposon-induced allele, *pie-1(zu177::Tc1)*, was obtained from a screen of ~500,000 animals[11], and was used to clone the *pie-1* gene (Fig. 2). A full-length *pie-1* complementary DNA sequence was used to search for related products in the protein and nucleic acids databases (Fig. 2). The *pie-1* gene encodes a novel protein, but has two copies of a motif originally described in the TIS-11/NUP475 family of proteins[4–7] (Fig. 2). This motif consists of a pattern of three cysteine and one histidine residues (Fig. 2*b*) and has been proposed to form a zinc-binding domain, or finger[4]. This motif may have an ancient origin; similar sequences are found in many animal, plant and fungal proteins, and are present in the predicted products of at least 10 genes identified by the *C. elegans* genome sequencing project (C.C.M., unpublished observations). Examples include the mammalian protein U2AF35 (ref. 12) and the *Drosophila* proteins Suppressor of Sable[13] and Unkempt[14]. Although U2AF35 and Suppressor of Sable have been implicated in pre-messenger RNA splicing, a biochemical function has not been established for this motif.

The *pie-1* mRNA is expressed maternally and is detected in gonads, oocytes and in all blastomeres until the four-cell stage of embryogenesis; during later stages the *pie-1* mRNA is degraded in somatic blastomeres but retained in the germline blastomeres (G. Seydoux, personal communication). This distribution has been reported previously for several other unrelated mRNAs in the early *C. elegans* embryo, and may represent the general pattern of degradation for non-localized maternal mRNAs[15].

To examine the distribution of the PIE-1 protein we raised antibodies against three different PIE-1-specific peptides (Fig. 3). Sera against all three peptides reveal a similar embryonic and mitotic distribution of PIE-1 protein. PIE-1 protein first becomes detectable at low levels in the posterior cytoplasm of one-cell stage embryos (data not shown). In two-cell stage embryos, PIE-1 is

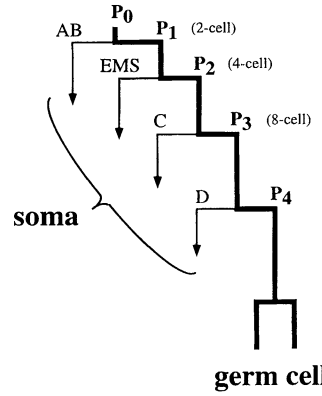

FIG. 1 The origin of germ cells in *C. elegans*, beginning with the fertilized egg (P_0). Each of the early divisions, represented by horizontal bars, produces a somatic blastomere (AB, EMS, C or D) and a germline blastomere (P_1, P_2, P_3 or P_4). Subsequent divisions of the somatic blastomeres are not shown.

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present in the nucleus and cytoplasm of the germline blastomere P_1, but at low or non-detectable levels in the somatic blastomere AB (Fig. 3*a*). In four-cell stage embryos, PIE-1 is present in P_2, but at low or non-detectable levels in its somatic sister EMS (Fig. 3*b*). As described previously[1], the SKN-1 protein is present in P_1, but when P_1 divides SKN-1 is present at equal levels in both of the P_1 daughters, EMS and P_2. Thus the asymmetrical localization of PIE-1 to P_2 nucleus provides a simple explanation of the observation that *pie-1*(+1) activity seems to prevent the activity of the SKN-1 transcription factor in P_2, but not in EMS[3]. PIE-1 protein remains localized to the germline blastomeres at each of the subsequent cleavages: In 8-cell and 16-cell stage embryos PIE-1 is detected only in P_3 and P_4, respectively (Fig. 3*c*, *d*). When P_4 divides, both of its daughters are germ-cell precursors, and PIE-1 is detected in the nuclei of both daughters (Fig. 3*e*).

PIE-1 appears to be associated with punctate structures in the cytoplasm of germline blastomeres (Fig. 3). Double-labelling experiments indicate that these structures are P granules[16], which are germline-associated particles of unknown function and composition (data not shown). P granules are present at all stages of the *C. elegans* life cycle in either mitotic germ cells (larvae), mature germ cells (oocytes in adults), or in germline blastomeres (in embryos)[16]. In contrast, PIE-1 is found in P granules only in germline blastomeres, beginning with the P_1 blastomere in the two-cell stage embryo.

Because the *pie-1* mRNA is present in all blastomeres in two-cell- and four-cell-stage embryos, translational or post-translational mechanisms must control the asymmetrical distribution of PIE-1 protein during these stages. For example, the association of PIE-1 with P granules during the early cell divisions could serve to localize PIE-1 to the germline blastomeres. A second possible contribution to PIE-1 asymmetry is suggested by the finding that PIE-1 antisera specifically stain the centrosomes of dividing germline blastomeres (Fig. 4). We have examined the intracellular distribution of PIE-1 as each germline blastomere divides, and find a common sequence of events. When germline blastomeres begin to divide, the nascent mitotic spindle complex rotates by roughly 90° (ref. 17). Before rotation, PIE-1 accumulates at apparently equal levels around each centrosome of the spindle, and PIE-1 staining diminishes in the nucleus, cytoplasm and P

a

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MAQTKPIAEQMAALNNSDDTSFAADRSNSLLNATCPARIQNSVDQRKINRSFNDSLSSGY -60
SGKWLRPKREALKITPLAQIDEAPATKRHSSAKDKHTEYKTRLCDAFRREGYCPYNDNCT -120
YAHGQDELRVPRRRQEYYSRDPPRERRDSRSRRDDVDTTINRSSSSASKHHDENRRPSNN -180
HGSSNRRQICHNFERGNCRYGPRCRFIHVEQMQHFNANATVYAPPSSDCPPPIAYYHHHP -240
QHQQQFLPFPMPYFLAPPPQAQQGAPFPVQYIPHQHDLMNSQPMYAPMAPTYYYQPINSN -300
GMPMMDVTIDPNATGGAFEVFPDGFFSQPPPTIIS                           -335
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b

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PIE-1    Y K T R L C D A F R R E G Y C P Y N D N C T Y A H -123
NUP475   Y K T E L C R T Y S E S G R C R Y G A K C Q F A H -120
U2AF35   K Y R P S C P F Y N K T G A C R F G N R C S R K H -181
su(S)    R K L E L C K F Y L M - D C C A K R D K C S Y M H -354
unkem    Y K T E P C K R P P R - - L C R Q G Y A C P Q Y H -244

PIE-1    N R R Q I C H N F E R - G N C R Y G P R C R F I H -208
NUP475   Y K T E L C H K F Y L Q G R C P Y G S R C H F I H -158
U2AF35   W K V A I C G L F E M - Q K C P K G K H C N F L H -321
su(S)    H K E F P C K Y Y Y L G M D C Y A G D D C L F Y H -379
unkem    Y K S T K C N D V Q Q A G Y C P R S V F C A F A H -324
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FIG. 2 The *pie-1* locus. *a*, Deduced amino-acid sequence from the nucleotide sequence of a 1,105-bp *pie-1* cDNA clone. Nucleotides 529 to 746 are coding sequences deleted in *pie-1(zu127)* (data not shown). *b*, Alignment of the *pie-1* zinc-finger motifs with sequences from nup475 (ref. 4), U2AF35 (ref. 12) and unkempt[14].

METHODS. The spontaneous mutation *pie-1(zu177::Tc1)* was obtained in a previously described genetic screen[11]. The mutant strain was outcrossed several times and placed over a chromosome marked with *nob-1(ct230)* and *unc-25(e156)* which flank *pie-1*. Recombinant animals yielded a map order of *nob-1*–32–*pie-1*–37–*unc-25*, and in all recombinants a single novel Tc1 transposon co-segregated with the *pie-1* mutation. The *C. elegans* genomic DNA flanking this transposon was isolated as described[24]. Probes were prepared and used to isolate four full-length cDNA clones; each cDNA contained the trans-spliced leader sequence SL1 (ref. 25) at its 5′ end and a poly(A) tail of varying length at its 3′ end. Three lines of evidence indicate that the open reading frame encodes the PIE-1 protein: The *pie-1(zu177::Tc1)* and *pie-1(zu127)* mutations are a transposon insertion and a deletion, respectively, in the genomic DNA corresponding to this open reading frame; *in vitro* transcribed RNA from these sequences, prepared as described[2,26], can induce a *pie-1* phenocopy when microinjected into wild-type animals; and antibodies raised against peptides deduced from this sequence recognize a protein that is present in wild-type embryos but absent in *pie-1* mutants (Fig. 3 and data not shown).

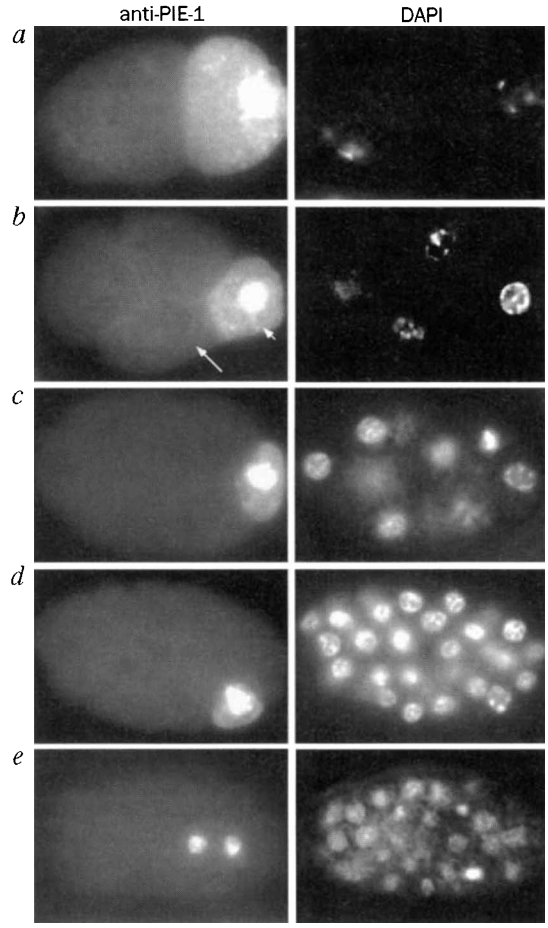

FIG. 3 PIE-1 localization in germline blastomeres. Each row represents a single early embryo stained with an antibody against the PIE-1 protein (left column) or with the dye DAPI to visualize nuclei (right column). *a*, Late two-cell stage embryo showing PIE-1 in the nucleus and cytoplasm of the P_1 blastomere. Faint, punctate staining in the cytoplasm of P_1, and all other germline blastomeres shown corresponds to P granules. *b*, An early four-cell stage embryo with PIE-1 in the nucleus and cytoplasm of the P_2 blastomere (short arrow). Faint PIE-1 staining is detected in the P_2 sister, EMS (long arrow). *c*, 12-cell stage embryo showing PIE-1 in the P_3 blastomere. *d*, 46-cell stage showing PIE-1 in the P_4 blastomere. *e*, 88-cell stage showing PIE-1 in the two P_4 daughters. The P_4 daughters do not divide again during embryogenesis, and in postembryonic development produce only germ cells (sperm and oocytes). PIE-1 is not detected in embryos after roughly 200 min of development. Embryos are 45 μm in length.

METHODS. The PIE-1 protein sequence shown in Fig. 2*a* was used to generate three peptides: A (residues 54–73), B (residues 81–105) and C (residues 134–157). Rabbit polyclonal antisera were generated against the individual peptides. Embryos shown were fixed and stained with antisera generated against peptide C, and with DAPI to visualize nuclei, and examined under the fluorescence microscope[2]. Similar staining results were obtained with each of the peptide antisera. PIE-1 staining with the antiserum against peptide C is not detectable in the nucleus, cytoplasm or P granules of embryos from mothers homozygous for the *pie-1* (zu127) mutation.

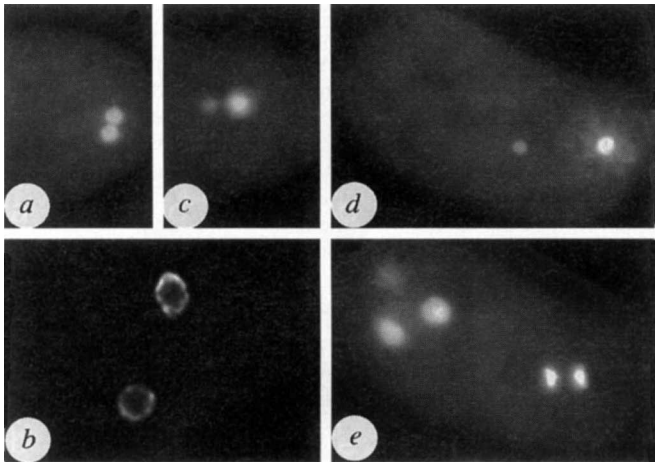

FIG. 4 Centrosomal localization of PIE-1 during division of the germline blastomere P_1. All images except e are of embryos stained with an affinity-purified antibody against PIE-1 peptide A and viewed with either a standard fluorescence microscope (*a*, *c*, *d*, *e*) or an optical sectioning microscope (*b*). *a*, Two-cell stage embryo (only the P_1 blastomere is shown). P_1 is at prophase; PIE-1 is not detected in the nucleus (not visible) but is detected prominently in the two centrosomes, which are shown here just before rotation of the centrosome–spindle complex (compare with the nuclear staining of the interphase P_1 blastomere in Fig. 3*a*). *b*, High-magnification image of PIE-1 staining at the P_1 centrosomes. *c*, The P_1 blastomere after rotation of the centrosome–spindle complex. The anterior (left) centrosome stains less intensely than the posterior (right) centrosome. *d*, Division of the AB and P_1 blastomeres (compare with DAPI image shown in e). The mitotic spindles of AB and P_1 have elongated; both centrosomes associated with the P_1 spindle have detectable PIE-1 staining (arrows), although the posterior centrosome (destined for the germline blastomere P_3) shows a much higher level of staining than the anterior centrosome (destined for the somatic blastomere EMS). Neither of the centrosomes associated with the dividing AB blastomere have detectable PIE-1 staining. *e*, DAPI image of embryo shown in *d*. The two nascent daughter nuclei of the dividing AB blastomere are visible on the left, and the two nascent daughter nuclei of the dividing P_1 blastomere are visible on the right.
METHODS. Identification of the PIE-1-containing structures shown here as centrosomes is based on co-staining experiments with an antibody against beta-tubulin[17]; in such experiments the PIE-1-containing structures are localized at the centre of each spindle aster (data not shown). Embryos were prepared and stained as described in Fig. 3. Image in *b* was collected on a Deltavision SA3.1 wide-field deconvolution optical sectioning microscope (Applied Precision) The image was deconvolved using the reiterative constrained method[27].

granules (Fig. 4*a*, *b*). After spindle rotation, the level of PIE-1 staining in one of the two centrosomes diminishes rapidly and becomes undetectable (Fig. 4*c*, *d*). Thus at the completion of cell division, only one daughter cell contains PIE-1 at its centrosome, and that daughter invariably is the new germline blastomere (P_1, P_2, P_3 or P_4). When the P_4 blastomere divides, PIE-1 staining persists in both centrosomes (data not shown).

Although we do not know the behaviour of individual PIE-1 molecules during the cell cycle, one model is that the PIE-1 protein translocates to centrosomes at cell division. Other proteins, such as NuMA (ref. 18) and CP190 (ref. 19), have been described previously that seem to cycle between the nucleus and centrosomes. We are not aware of proteins other than PIE-1 that disappear from one of the two centrosomes, and thus become distributed asymmetrically after cell division.

What might distinguish the two centrosomes in a dividing germline blastomere? In a study on the control of spindle orientation in *C. elegans*, the relative positions of the two centrosomes of a germline blastomere were interchanged before rotation of the mitotic spindle was complete[20]. These manipulated embryos developed normally, suggesting that both centrosomes initially are equivalent. Thus we propose that rotation of the mitotic spindle in the germline blastomeres may bring the centrosomes into different intracellular environments that affect PIE-1 stability or binding. The idea that asymmetry exists within each germline blastomere is supported by the recent finding that P granules also seem to be degraded or unstable in the pre-somatic 'half' of a germline blastomere before division[21].

The early divisions of the *C. elegans* embryo represent a classic stem-cell-like lineage in which one pluripotent cell gives rise sequentially to daughters with distinct, and more restricted, developmental potentials. In *C. elegans*, the fate of the first somatic blastomere, AB, is determined in part by responses to external cell signals[11,22]. As described above, the fate of the second somatic blastomere, EMS, is determined at least in part by two transcription factors that are present in both P_2 and its sister cell EMS[1,2]. It is likely that differentiation in stem cell lineages in other animals is also determined by several different intercellular or intracellular signals, and that the pluripotent cells must either not contain, or not respond to, these signals.

The PIE-1 protein in *C. elegans* provides an example of how the pluripotent cell in a stem cell lineage might be protected from the signals that promote differentiation in other cells. We have shown here that the PIE-1 protein is localized to the totipotent germline blastomere after each division in the early embryo. This localization correlates with the repression of somatic cell fates[3], and also seems to correlate with a general repression of transcription within the germline[23]. Thus PIE-1 may represent a localized general repressor that serves to antagonize the activity of a more broadly expressed set of transcriptional activators. This may provide an efficient means for generating diversity within a stem cell lineage: if cells in a stem cell lineage express determinative molecules at different times or in response to different signals, there need not be separate mechanisms for segregating these molecules away from the pluripotent stem cell. Instead, a single mechanism for localizing a general repressor at each division could maintain the pluripotency of the stem cell. □

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1. Bowerman, B., Draper, B. W., Mello, C. M. & Priess, J. R. *Cell* **74,** 443–452 (1993).
2. Lin, R., Thompson, S. & Priess, J. R. *Cell* **83,** 599–609 (1995).
3. Mello, C. C., Draper, B. W., Krause, M., Weintraub, H. & Priess, J. R. *Cell* **70,** 163–176 (1992).
4. Dubois, R. N., Mclane, M. W., Ryder, K., Lau, L. F. & Nathans, D. *J. biol. Chem.* **265,** 19185–19191 (1990).
5. Gomperts, M., Pascall, J. C. & Brown, K. D. *Oncogene* **5,** 1081–1083 (1990).
6. Varnum, B. C., Ma, Q., Chi, T., Fletcher, B. & Herschman, H. R. *Molec. cell. Biol.* **11,** 1754–1758 (1991).
7. Bustin, S. A. *et al. DNA Cell Biol.* **13,** 449–459 (1994).
8. Sulston, J. E., Schierenberg, E., White, J. G. & Thomson, J. N. *Devl Biol.* **100,** 64–119 (1983).
9. Bowerman, B., Eaton, B. A. & Priess, J. R. *Cell* **68,** 1061–1075 (1992).
10. Blackwell, T. K., Bowerman, B., Priess, J. R. & Weintraub, H. *Science* **266,** 621–628 (1994).
11. Mello, C. C., Draper, B. W. & Priess, J. R. *Cell* **77,** 95–106 (1994).
12. Zhang, M., Zamore, P. D., Carmo-Fonseca, M., Lamond, A. I. & Green, M. R. *Proc. natn. Acad. Sci. U.S.A.* **89,** 8769–8773 (1992).
13. Voelker, R. A., Gibson, W., Graves, J. P., Sterling, J. F. & Eisenberg, M. T. *Molec. cell. Biol.* **11,** 894–905 (1991).
14. Mohler, J. *et al. Genetics* **131,** 377–388 (1992).
15. Seydoux, G. & Fire, A. *Development* **120,** 2823–2834 (1994).
16. Strome, S. & Wood, W. B. *Proc. natn. Acad. Sci. U.S.A.* **79,** 1558–1562 (1982).
17. Hyman, A. A. & White, J. G. *J. Cell Biol.* **105,** 2123–2135 (1987).
18. Frash, M., Glover, D. M. & Saumweber, H. *J. Cell Sci.* **8,** 467–480 (1986).
19. Oegema, K., Whitfield, W. G. F. & Alberts, B. *J. Cell Biol.* **131,** 1261–1273 (1995).
20. Hyman, A. A. *J. Cell Biol.* **109,** 1185–1193 (1989).
21. Hird, S. N. *et al. Development* **122,** 1303–1312 (1996).
22. Bowerman, B. *Bioessays* **17,** 405–414 (1995).
23. Seydoux, G. *et al. Nature* **382,** 713–716 (1996).
24. Hill, R. & Sternberg, P. *Nature* **358,** 470–476 (1992).
25. Krause, M. & Hirsh, D. *Cell* **49,** 753–761 (1992).
26. Guo, S. & Kemphues, K. J. *Cell* **81,** 611–620 (1995).
27. Hiraoka, Y., Swedlow, J. R., Paddy, M. R., Agard, D. A. & Sedat, J. W. *Semin. Cell Biol.* **2,** 153–165 (1991).

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